Inhibition of protein synthesis by the human immunodeficiency virus type 1 nef gene product

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During productive infection of human T lymphocytes in cell culture, the expression of human immunodeficiency virus type 1 is temporally regulated by virus-encoded regulatory proteins. Among these Nef, whose function has not been clearly elucidated, is thought to alter CD4+ T cells. We examined the possibility that the nef gene interferes with the translation process in a cell-free system. The results demonstrate that the nef gene product mediates an inhibitory effect on protein synthesis. Conversely, the use of antisense nef mRNA did not affect translation. Further observations suggest that this inhibitory effect is an inherent property of the nef gene product itself and not of its mRNA. The data show that the translational repression directed by Nef is a general phenomenon, acting on its own and on other messengers used as reporter mRNAs. We propose that, as a consequence, Nef can play an important role in the pathogenesis of AIDS.

Introduction

The human immunodeficiency virus type 1 (HIV-1) Nef protein is one of three viral proteins synthesized from spliced mRNAs early during infection and is dispensable for virus growth in T cell lines. The nef gene from the HIV-1SF2 strain has been shown to down-regulate HIV-1 expression (Cheng-Mayer et al., 1989; Luciw et al., 1987), modulate cell surface expression of the CD4 molecule (Garcia & Miller, 1991; Garcia et al., 1993; Schwartz et al., 1993) and interfere with a signal emanating from the T cell receptor complex with subsequent blocking of interleukin-2 mRNA synthesis (Luria et al., 1991). In addition, the expression of Nef from the SF2 isolate in transgenic mice has been shown to perturb development of CD4+ T lymphocytes (Skowronski et al., 1993). These observations suggest that Nef plays an important role in the virus infection cycle through a cellular activity and hampers the maturation of the T cell population as well. However, the mechanism by which nef gene expression alters CD4+ T cell state has not been elucidated. We have used a cell-free translation system to explore the possibility that the HIV-1SF nef gene product interferes with the translation machinery. The results obtained by the use of various indicator mRNAs demonstrate that Nef exhibits a strong inhibitory activity on protein synthesis.

Methods

Plasmids. The DNA fragment encoding HIV-1SF2 nef was inserted in the Bluescript KS(+) vector (Stratagene) as previously described (Poulin & Levy, 1992). In addition, a GC-rich region (Sac1I-PstI) from the Bluescript plasmid containing nef (pBS2 nef) was removed to avoid interference during the translation from any putative secondary structure present in the generated mRNA. A second nef construct (pGEM-3 nef) was made by subcloning an EcoRI-HindIII fragment of the modified pBS2 nef plasmid in the same site of the multiple cloning site of the pGEM-3 vector (Promega). An additional construct was prepared by inserting the linker 5'TAGTCTGACTAG3' in the unique Xhol site of the nef gene. This linker contains amber stop codons in the three reading frames. Site directed mutagenesis (Kunkel, 1985) was also used to create a nef mutant. By using the synthetic oligonucleotides 5'GGAATTCGATCACGCGTGGCAAGTGGT3', 5'CAAAACGTAGTACTGGTGGATGGTCT3' and 5'TATAAG-GGAACGATCGAGACGAGCTGA3', the three putative initiator codons were changed. The resulting mutant nef gene was transferred into the pGEM-3 vector as described above.

pGEM-3 CAT was constructed by inserting a HindIII-BamHI fragment containing the entire coding sequence of the chloramphenicol acetyltransferase (CAT) gene from pSV2 CAT into pGEM-3.

In vitro transcription/translation. After linearization with appropriate restriction enzymes, plasmids were used as templates for transcription by T7 or SP6 polymerase as previously indicated (Poulin & Levy, 1992). When mRNA was to be visualized on a gel, [3H]CTP (Amersham; 20 Ci/mmole) was used in the mixture at 1 mCi/ml. Globin and luciferase mRNAs were obtained from BRL and Promega respectively. In vitro translations of the various mRNAs were performed in micrococcal nuclease-treated rabbit reticulocyte lysates as described by the manufacturer (Promega) using [35S]-methionine (Amersham; 1200 Ci/mmole) at 1 mCi/ml. Translation
products were analysed by SDS-PAGE. Gels were treated with ENHANCE (NEN), dried and exposed to X-ray film at -70 °C.

In order to verify mRNA integrity, aliquots taken from the translation mixture were digested with proteinase K (400 μg/ml), phenol extracted, ethanol precipitated and analysed on a 1.5% formaldehyde-agarose gel. The gel was treated with ENHANCE, dried and exposed to X-ray film at -70 °C.

Results and Discussion

nef constructs

To study the effect of nef on the translation process, different constructs were prepared as shown in Fig. 1. The HIV-1 nef gene was inserted into pBluescript

(a) pBluescript-nef

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1. T7 promoter

GCGGAAUUGGAGCTCCACCGGAAUUGGAGCUG...

2. T7 promoter

GCGGAAUUGGAGCTCCACCGGAAUUGGAGCUGA...

(b) pGEM-3 nef

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1. T7 promoter

GCGGAAUUGGAGCTCCACCGGAAUUGGAGCUG...

2. SP6 promoter

GAAUACA

3' end of the nef fragment

(c) pGEM-3 CAT

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SP6 promoter

GAAUACAGCUUGGCGAATUUUAGGACUAAAGAAA

GAAUACAG...

Fig. 1. Plasmid constructs used to study the effect of nef mRNA on the translation process. (a) pBluescript-nef; (b) pGEM-3 nef; (c) pGEM-3 CAT. Sequences shown correspond to the 5' regions of the mRNAs synthesized by T7 or SP6 RNA polymerases between the promoter sequence and the first AUG on the corresponding mRNA.

downstream of the T7 promoter. The sequence generated following transcription includes an untranslated region of 77 nucleotides [Fig. 1a(1)]. The resulting mRNA was translated in reticulocyte lysate and in wheat germ extracts. However, the level of synthesis was very low in wheat germ extract; we therefore used reticulocyte lysate for our studies. In this translation system, the efficiency of the nef mRNA appeared to be 10-fold less than that of two other mRNAs (globin and luciferase) used as controls under similar conditions. In order to maximize the translation efficiency, the length of the region preceding the first AUG of the nef sequence was reduced by removing a GC-rich region that tends to result in the formation of secondary structures. The new transcript obtained has a 5' untranslated region of 30 nucleotides [Fig. 1a(2)]. In spite of this modification, the translation efficiency of the nef mRNA was still very low. As a control a second transcription vector, pGEM-3, was used to generate nef mRNA using T7 polymerase. The 5' untranslated region resulting from the T7 RNA polymerase-driven transcription is 19 nucleotides long [Fig. 1b(1)]. The translation efficiency was similar to that obtained with a longer 5' untranslated region (above), suggesting that the nature of the nef mRNA and/or its product can play a determining role in the translation efficiency rather than the length of the untranslated region itself. Moreover, addition of fresh nef mRNA to a lysate that had already been incubated for 15 min in the presence of nef message did not result in any further increase in the amount of p27 nef synthesized.

An antisense nef mRNA was also prepared from pGEM-3 nef using the opposite SP6 promoter. The resulting transcript is complementary to the RNA synthesized by T7 polymerase with the exception of the first seven nucleotides [Fig. 1b(2)]. This mRNA does not generate any detectable product following translation. In contrast, very high synthesis efficiency was obtained when the CAT sequence was translated from mRNA obtained with the same pGEM-3 vector using SP6 polymerase, and with a 42-nucleotide-long sequence before the first AUG (Fig. 1c). Altogether, this information prompted us to investigate whether the nef gene and/or the nef gene product could affect the translation process.

Effect of nef mRNA on the translation process

Rabbit globin mRNA was translated in the presence of nef mRNA in a rabbit reticulocyte lysate. Although the simultaneous addition of both mRNAs did not affect the translational efficiency of each message early in the translation assay, a sharp decrease in the global rate of synthesis took place after 10 min incubation (data not shown). However, when the globin mRNA was added to
Translational repression by Nef

Fig. 2. (a) Inhibition of globin mRNA translation in the presence of nef mRNA: SDS–PAGE of [35S]methionine-labelled translation products. Globin mRNA was translated in a reticulocyte lysate preincubated for 15 min in the absence (globin) or in the presence (nef+globin panel) of nef mRNA. Translation was performed using nef mRNA obtained from the transcription of modified pBS2 nef, at a concentration of 20 μg/ml and globin mRNA (BRL) at 10 μg/ml. Aliquots of [35S]methionine-labelled products were removed at the indicated time points and electrophoresed on a 12% SDS–polyacrylamide gel. The upper gel was exposed for 4 days and the bottom gel for 4 h. (b) Stability of translation products. The stability of globin synthesized over a 15 min period in a reticulocyte lysate preincubated for 15 min in the absence (globin) or presence (nef+globin) of nef mRNA was analysed after addition of cycloheximide (cyx) at 10 μg/ml by measuring [35S]methionine incorporation in aliquots removed at different time intervals and TCA-precipitated. Background level, as measured for a minus mRNA translation reaction, has been subtracted for each time point. (c) Globin synthesis in the presence of the p27 nef protein. Reticulocyte lysate preincubated for 15 min in the absence or presence of 3H-labelled nef mRNA was nuclease-treated to degrade mRNA, diluted with an equal volume of fresh lysate, incubated for another 10 min and used to direct the translation of globin mRNA. ([35S]Methionine incorporation was measured in aliquots removed at different time intervals. Time 0 corresponds to the addition of globin mRNA. Globin, lysate preincubated for 15 min without nef mRNA. nef+globin, lysate preincubated for 15 min in the presence of nef mRNA. In the gel, aliquots of 3H-labelled nef mRNA were run on a formaldehyde gel following incubation of the translation mixture in the absence (lane 1) or presence (lane 2) of micrococcal nuclease. Molecular size standards (BRL) are indicated. To remove nef mRNA from the translation mixture, micrococcal nuclease was added to a final concentration of 120 U/ml in the presence of 1 mM-CaCl₂, incubated for 15 min at 25 °C and inactivated by 2 mM-EGTA.

The translation mixture following preincubation of nef mRNA for a period of 15 min to allow sufficient Nef synthesis, there was a marked inhibition (around 95%) in the amount of globin produced (Fig. 2a, nef+globin). In the absence of nef mRNA, the preincubation step did not affect globin synthesis, which proceeded linearly for at least 30 min (Fig. 2a, globin). We determined that for immediate inhibition of the amount of protein synthesized, the nef mRNA had to be added at least 10 min before the second mRNA. This delay suggests that a biochemical event resulting from the presence of Nef might be involved in the apparent inhibition of translation.

To rule out the possibility that the low level of globin
Fig. 3. (a) Effect of sense and antisense nef mRNA on luciferase mRNA translation. Sense and antisense oriented nef mRNAs were generated in vitro using T7 and Sp6 polymerase according to the appropriately positioned promotors of the pGEM-3 vector. Luciferase mRNA (Promega) was translated in a reticulocyte lysate preincubated for 15 min with antisense nef mRNA (antisense nef+ Luc) or sense nef mRNA (sense nef+ Luc) and without any nef mRNA (Luc). For the translation, both sense and antisense nef mRNAs were preincubated for 15 min at 20 µg/ml; the luciferase mRNA was then added at a final concentration of 20 µg/ml. Reticulocyte lysate mixture preincubated without any mRNA before adding luciferase mRNA was used as a control. The kinetics of luciferase synthesis are shown. Aliquots of [35S]methionine-labelled product were removed at the indicated time points following the preincubation step with the first mRNA and electrophoresed on a 12% SDS-polyacrylamide gel. Gels showing luciferase synthesis in the presence of the antisense nef mRNA (left panel) and in the absence of any mRNA (right panel) were exposed for 4 h. The antisense nef mRNA preincubated for 15 min before adding luciferase mRNA did not produce any detectable stable product by TCA-precipitable counts. The gel which does not show any synthesis of luciferase in the presence of nef mRNA preincubated for 15 min before adding luciferase mRNA was exposed for 48 h (middle panel). (b) Luciferase mRNA translation in the presence of globin mRNA. Both globin and luciferase mRNAs were used at a final concentration of 20 µg/ml. Luciferase mRNA was added to a reticulocyte lysate mixture containing globin mRNA preincubated for 15 min (globin + Luc) or without any globin mRNA (Luc). Continuation of globin synthesis after preincubation for 15 min of its mRNA without addition of any message is shown in the middle panel (globin). (c) Luciferase mRNA translation in the presence of a mutated nef mRNA. Luciferase mRNA was translated in reticulocyte lysate preincubated for 15 min with nef mRNA mutated at the three initiator AUGs and containing a stop codon (1-2-3 ATG/SC + Luc), with an antisense nef mRNA (antisense nef + Luc) or in the absence of any nef mRNA (Luc). Translation of the nef mutant (1-2-3 ATG/SC) as well as of antisense nef mRNAs alone are shown. A blank reaction without any mRNA was carried out (negative control). Reactions of luciferase synthesis were done as for (a).
detected was due to a decrease in the rate of protein synthesis or to a reduction in the stability of the protein, cycloheximide, a protein synthesis inhibitor, was added to the translation reaction after 15 min of globin synthesis. Aliquots were then removed at different time periods and TCA-precipitable counts were measured for each aliquot. Fig. 2(b) shows that during the 35 min following addition of cycloheximide, there was no substantial change in the amount of protein, whether the lysate was incubated in the absence or in the presence of nef mRNA. This result indicates that the observed decrease in the amount of globin synthesized is not the result of a differential stability of the translated product, but reflects a decrease in the level of protein synthesis following preincubation of the lysate with nef mRNA.

To further evaluate whether the observed inhibition was due to competition between both mRNAs present in the translation mixture rather than the presence of the Nef protein itself, the lysate was treated with micrococcal nuclease at the end of the preincubation step in order to remove completely nef mRNA. As expected, the nuclease treatment totally degraded nef mRNA (Fig. 2c, gel). After the nuclease treatment, fresh lysate was added and the incubation allowed to proceed for 10 min. The globin message was then added and aliquots taken during the next 30 min. Fig. 2(c) shows that there was no new globin synthesis when the fresh lysate and globin mRNA were incubated in the lysate that had been preincubated with nef mRNA (Fig. 2c, nef+globin). This finding contrasts with the linear increase in the amount of globin (TCA-precipitable counts) detected when the globin mRNA was translated in the nuclease-treated reticulocyte lysate that had not previously contained any nef mRNA (Fig. 2c, globin). The total amount of globin synthesis was however reduced in this assay (compare Fig. 2b and 2c, globin) since the final concentration of globin mRNA was diluted by a factor of two following the addition of fresh lysate. These results demonstrate that a strong inhibition (around 92%) of globin synthesis persists in the presence of the Nef protein alone and is not directly dependent on the presence of nef mRNA.

**Effect of an antisense nef mRNA on the translation process**

To substantiate the negative effect of the nef gene product on the translation process, we explored the use of antisense nef mRNA. Fig. 3(a) shows the kinetics of luciferase synthesis when either antisense or sense nef mRNAs were preincubated before adding luciferase indicator mRNA. In that case, luciferase synthesis was completely abrogated in the presence of sense nef mRNA (sense nef+Luc) indicating a very strong inhibition of translation (> 99%). Conversely, the antisense nef mRNA did not have any substantial effect on luciferase translation (antisense nef+Luc) as compared to the luciferase gene translated in the absence of any other messenger (Luc). From these experiments, the probability that the presence of contaminating dsRNAs coming from the in vitro transcription preparations interferes with the translation process is low since antisense nef was prepared in a similar way to the sense nef mRNA.

The results obtained from these experiments were reproducible with many preparations of plasmid DNA and in vitro transcription assays. As both sense and antisense nef mRNAs are likely to have similar specific stem-loops, the differential effect obtained by using both mRNAs does not seem to be assignable to these features. In a complementary control experiment where globin and luciferase mRNAs replaced nef mRNA under the same experimental conditions including a preincubation step with the first mRNA used, no such strong inhibitory effect was observed (Fig. 3b). Moreover, luciferase synthesis was not interrupted in the presence of globin mRNA and vice versa (globin+Luc). However, a slight decrease in the translation level of both mRNAs was observed. This effect is not significant with regard to the inhibitory effect obtained in the presence of nef mRNA and might be attributed to some degree of competition between both mRNAs.

In the same line, we have generated a nef mutant. When a stop codon was introduced at the unique XhoI restriction site of the nef sequence leaving intact the synthesis of the first 39 residues, the inhibition of protein synthesis was preserved (data not shown). Additional modification was performed at each of the three initiator ATGs by site directed mutagenesis. With these mutations, no nef gene product could be synthesized (Fig. 3c, 1-2-3 ATG/SC). This aberrant nef mRNA was used to test whether the effect on luciferase synthesis could be revoked. As expected, synthesis of luciferase was not repressed in the presence of such a nef mutant (Fig. 3c, 1-2-3 ATG/SC+Luc). However, the level of luciferase synthesis was lower than in the presence of antisense mRNA (Fig. 3c, antisense nef+Luc). These results support the conclusion that the inhibitory effect of Nef on protein synthesis is associated with the nef gene product activity.

**Effect of nef mRNA translation on the stability of a reporter mRNA**

To further characterize the inhibition of translation induced by Nef, the same experiments were repeated under similar conditions using chloramphenicol acetyltransferase (CAT) as a reporter mRNA. Fig. 4(a) shows
Fig. 4. Inhibition of CAT synthesis in the presence of nef mRNA. CAT mRNA was generated under the control of the SP6 promoter and translated at 5 μg/ml in a reticulocyte lysate preincubated for 15 min in the absence (CAT) or presence (nef+CAT) of nef mRNA. (a) [35S]Methionine incorporation was measured in aliquots removed at different time intervals. Time 0 corresponds to the addition of CAT mRNA. [35S]Methionine incorporation during nef mRNA translation (nef) or in the absence of nef mRNA (−mRNA) translation are shown as controls. (b) SDS-PAGE of [35S]methionine-labelled translation product. Aliquots removed 10 min after addition of CAT mRNA were electrophoresed on a 12% polyacrylamide gel. (c) Stability of CAT mRNA. In order to verify mRNA stability, mRNA-containing aliquots were taken out at different time intervals after addition of [3H]-labelled-CAT mRNA. Markers (BRL) are shown to the left.
double-stranded RNA-dependent protein kinase (dsRNA-PK) in vitro and in vivo results in subsequent phosphorylation of eIF-2α with consequent inhibition of protein synthesis (Hovanessian, 1991; Koromilas et al., 1992). Studies examining physiological functions of the kinase suggest that it participates in cell growth and differentiation by regulating protein synthesis. Furthermore, the dsRNA-dependent kinase is thought to be a key mediator of the antiviral effect and antiproliferative effects of interferon (Sen & Lengyel, 1992). In our studies, it is possible that the presence of Nef induces the activation of a kinase in the reticulocyte lysate, and leads to the inhibition of translation by analogous mechanisms. Recently, the HIV-1sp2 Nef expressed in cultured cells has been found associated with a cellular serine kinase activity that phosphorylates cellular proteins of 62 and 72K (Sawai et al., 1994).

Our previous results have shown that three Nef-specific polypeptides of 29, 27 and 25K can be produced in vitro, suggesting that these various forms are derived from different in-frame initiator ATGs (Poulin & Levy, 1992). Of these, only the product starting from the first ATG would be myristoylated (Kaminchik et al., 1991). In the light of a functional difference between Nef isoforms (Greenway et al., 1994), the first ATG was mutated to further investigate the role of myristoylation for the Nef inhibitory effect. Although the nef gene product derived from the first ATG can incorporate [3H]myristate in our assay, mutation of the first ATG did not revoke the observed inhibitory effect (data not shown). These results suggest that myristoylation of the Nef protein is not critical for the observed inhibition of translation, at least in our in vitro system.

Whereas the inhibition of translation processes by Nef has been demonstrated in an in vitro system, a similar effect of this viral protein in cultured cells remains to be investigated. The effect of Nef on thymocyte maturation in transgenic mice (Skowronska et al., 1993) does suggest that this protein could affect intracellular events such as those involved in activation. Similarly, the ability of Nef to inhibit translation as shown in these experiments could reflect an effect on important physiological events in the control of virus and host gene expression. Moreover, the influence of Nef on HIV mRNA translation may be responsible, in part, for the modulation by Nef of virus expression in cultured cells (Luciw et al., 1987; Terwilliger et al., 1986).

Although the inhibition of protein synthesis by Nef and the Nef-induced down-regulation of CD4 and IL-2 are probably mediated by different mechanisms, they may all be initiated by a common biochemical function with consequent pleiotropic effects on the host cell. Most importantly, an interference with cell growth by inhibition of protein synthesis could be involved in inducing latency during HIV infection. Moreover, it could affect the normal response of immune cells infected by the virus in the absence of direct cytopathic effect (Skowronska et al., 1993). Thus, impairment of CD4+ T cell function by nef through protein synthesis inhibition could have a direct impact on HIV pathogenesis.

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